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Effect of Modified Atmosphere Packaging on the Persistence and Expression of Virulence Factors of *Escherichia coli* O157:H7 on Shredded Iceberg Lettuce†

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ABSTRACT

Fresh-cut leafy greens contaminated with *Escherichia coli* O157:H7 have caused foodborne outbreaks. Packaging conditions, coupled with abusive storage temperatures of contaminated lettuce, were evaluated for their effect on the potential virulence of *E. coli* O157:H7. Shredded lettuce was inoculated with 5.58 and 3.98 log CFU *E. coli* O157:H7 per g and stored at 4 and 15°C, respectively, for up to 10 days. Lettuce was packaged under treatment A (modified atmosphere packaging conditions used for commercial fresh-cut produce, in gas-permeable film with N_2), treatment B (near–ambient air atmospheric conditions in a gas-permeable film with microperforations), and treatment C (high-CO_2 and low-O_2 conditions in a gas-impermeable film). *E. coli* O157:H7 populations from each treatment were determined by enumeration of numbers on MacConkey agar containing nalidixic acid. RNA was extracted from packaged lettuce for analysis of expression of virulence factor genes stx_2, *eae, ehxA, iha,* and *rfbE*. *E. coli* O157:H7 populations on lettuce at 4°C under all treatments decreased, but most considerably so under treatment B over 10 days. At 15°C, *E. coli* O157:H7 populations increased by at least 2.76 log CFU/g under all treatments. At 15°C, expression of *eae* and *iha* was significantly greater under treatment B than it was under treatments A and C on day 3. Similarly, treatment B promoted significantly higher expression of stx_2, *eae, ehxA,* and *rfbE* genes on day 10, compared with treatments A and C at 15°C. Results indicate that storage under near–ambient air atmospheric conditions can promote higher expression levels of O157 virulence factors on lettuce, and could affect the severity of *E. coli* O157:H7 infections associated with leafy greens.

Investigations of leafy green outbreaks over the last 5 years have focused attention on the contamination of these commodities with enterohemorrhagic *Escherichia coli* (EHEC), specifically *E. coli* O157:H7. From 1973 to 2006, 5% of foodborne outbreaks were attributed to contaminated leafy greens in the United States *(14)*. However, by 2007, contaminated leafy greens were responsible for 14% of identified foodborne outbreaks attributed to a single commodity, making them the leading produce commodity causing foodborne illness in the United States *(9)*. Infections of EHEC typically have incubation periods of 3 to 5 days, and patients experience nonbloody diarrhea and abdominal cramps *(24)*. In cases more severe, infection can progress to cause hemorrhagic colitis (bloody diarrhea) and move on to hemorrhagic uremic syndrome (HUS), which leads to kidney failure and thrombocytopenia *(27)*. The development of HUS has been attributed to the presence of Shigi toxin (Stx), produced by EHEC strains during infection *(27)*. Infections from outbreaks of *E. coli* O157:H7 associated with bagged, cut leafy green commodities have increased in severity over the past 5 years. Average hospitalization rates and incidences of HUS attributed to *E. coli* O157:H7 infections are estimated to be 29.5 *(21)* and 5 to 10%, respectively *(27)*. According to the Centers for Disease Control and Prevention, there were 199 reported cases of illness associated with the consumption of fresh spinach contaminated with *E. coli* O157:H7, of which 102 (51%) persons were hospitalized; 31 (16%) suffered from HUS *(6)*. Another multistate outbreak of *E. coli* O157:H7 infections associated with the consumption of contaminated shredded lettuce in 2006 resulted in 71 cases of illness, 53 (75%) persons hospitalized, and 8 (11%) cases of HUS *(7)*. In 2010, an outbreak of *E. coli* O145 (another EHEC) was associated with fresh-cut Romaine lettuce. This incident led to 30 cases of illness, of which 12 (40%) of the patients were hospitalized, and 3 (10%) developed HUS *(8)*. All three of these outbreaks were unusual because of the high rates of hospitalization and incidences of HUS. Strains of *E. coli* O157:H7 that possess the Stx2 gene (stx_2) are known to be more virulent than strains that possess stx_1 are *(1)*. Analysis of clinical isolates of *E. coli* O157:H7 from the 2006 spinach and lettuce outbreaks indicates that these strains possess a combination of stx_2 genes, which might

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have played a role in the increased virulence *(28)*. However, these strains did not exhibit exceptional phenotypic properties that would have prolonged their survival on leafy green commodities.

Potential causes of severe *E. coli* O157:H7 infections associated with fresh-cut leafy greens under various packaging and storage temperature conditions deserve investigation. Several other workers have addressed the possibility that *E. coli* O157:H7 and other bacterial pathogens might differently express virulence factors under varying conditions. A previous study with *E. coli* O157:H7 has shown that the expression of virulence factors differ in bovine colonization and human infections *(23)*. On leafy greens, stx_2 and intimin (*eae*) gene expression was slightly upregulated on Romaine lettuce inoculated with an *E. coli* O157:H7 strain expressing both stx_1 and stx_2 genes, stored at 4°C for 9 days, under atmospheric conditions *(5)*. Based on these findings, we postulate that the virulence of *E. coli* O157:H7 could be affected and possibly enhanced when persisting on iceberg lettuce under various packaging conditions. Our research objectives were to compare levels of expression of five virulence factor–encoding genes (*ehxA, eae, iha, rfbE,* and stx_2) of *E. coli* O157:H7 on shredded iceberg lettuce stored at 4 and 15°C, under three different packaging conditions, to determine if *E. coli* O157:H7 cells under these conditions could become ''primed'' to cause illness. Expression of these virulence factors was measured by real-time, reverse transcriptase PCR (RT-PCR) methods.

MATERIALS AND METHODS

Strain used. *E. coli* O157:H7 strain 52 (ATCC 43895), resistant to nalidixic acid and rifampin *(3),* was obtained from the U.S. Department of Agriculture, Agricultural Research Service, Environmental Microbial and Food Safety Laboratory culture collection, cultured on tryptic soy agar (TSA; BD, Franklin Lakes, NJ) supplemented with 50 μg/ml nalidixic acid, and incubated at 37°C for 24 h. Three colonies were inoculated into a sterile, fecal bovine manure slurry *(25),* incubated at 37°C for 24 h, and then isolated on MacConkey agar (Acumedia, Lansing, MI) supplemented 50 μg/ml nalidixic acid (MACN). A single colony was then inoculated into 200 ml of sterile manure slurry, agitated at 150 rpm, and incubated at 37°C for 48 h.

Preparation of shredded lettuce. Shredded iceberg lettuce from a commercial private label supplier in California was shipped overnight at 4°C under refrigerated conditions, and kept at 4°C until use. Shredded lettuce was weighed, and 500 g was placed into each of two sterile 5-liter beakers. Lettuce was then stored at 4°C for 20 min while the *E. coli* O157:H7 inoculum was prepared.

Preparation of inoculum. Inoculated manure slurry (40 ml) containing 8.26 CFU *E. coli* O157:H7/ml was transferred into a sterile, round-bottomed centrifuge tube. Inoculated slurry was centrifuged (Allegra 25R, Beckman-Coulter, Fullerton, CA) for 10 min at 5,000 × *g*. Supernatant was immediately decanted, and cell pellets were resuspended in 40 ml of sterile water. Cell suspensions were diluted 1:1,000 in 3,000 ml of sterile water, and agitated with a stir bar for 3 min before 500 g of lettuce was introduced into the cell suspension. The population of *E. coli* O157:H7 exposed to shredded lettuce was 4.81 log CFU/ml for lettuce stored at 15°C. For inoculated lettuce stored at 4°C, the original cell suspension was diluted 1:10 in 3,000 ml of sterile water so that the population of *E. coli* O157:H7 was 6.80 log CFU/ml.

Inoculation of shredded lettuce. Shredded lettuce (500 g) was added to 3,000 ml of inoculum in a 5-liter beaker. Lettuce was submerged and completely immersed in the inoculum for 30 min. The liquid inoculum was decanted by pouring through a sterile colander and collecting in a 4-liter beaker. Sterile tongs were used to aid the transfer of lettuce to the colander, and lettuce was then transferred to the basket of a salad spinner (OXO International, Ltd., Chambersburg, PA) sterilized with 70% ethanol and UV light, inside a biosafety cabinet. Remaining water was removed from shredded lettuce by spinning lettuce for 3 min in baskets in salad spinners. Baskets containing lettuce were then moved into a biosafety cabinet for packaging of lettuce.

Packaging of lettuce. Inoculated and uninoculated lettuce shreds were divided into 35-g portions and packaged in bags 9 by 14 cm, prepared from polyethylene films of different gas permeability and treatment conditions. Thirty bags containing 35 g of inoculated shreds and 30 bags containing the same amount of uninoculated lettuce were packaged in each replicate experiment. For treatment A, packages were prepared with a gas-permeable film with oxygen transmission rate (OTR) of 110 cc O_2/100 in^2/24 h (cm^3/m^2/24 h). The packages were flushed with N_2 to reach an initial headspace O_2 level of 2%, and sealed with a vacuum packaging machine (model C200, Multivac, Wolfertschwenden, Germany) placed in a biosafety hood. Packages for treatment B were prepared with the same gas-permeable film as was utilized in treatment A, but with 40 microperforations made with a 25-gauge syringe needle. Care was given to ensure that the perforations were uniformly distributed on the package film. For treatment C, the packages were prepared with gas-impermeable film (0 OTR) without any perforations. After sealing, all bags were stored at the chosen temperatures (4 and 15°C) for up to 10 days. Microbial populations were determined from the package samples during storage. Uninoculated lettuce was directly packaged from commercially supplied lettuce.

Nucleic acid extraction from shredded lettuce inoculated with *E. coli* O157:H7 and packaged under different gaseous atmospheric conditions. On days 1, 3, 7, and 10, bacterial RNA from *E. coli* O157:H7 on shredded lettuce packaged under treatments A, B, and C, stored at either 4 or 15°C, was extracted with a method based on that of Kyle et al. *(15)*. A single bag for each treatment at each temperature was analyzed. Sterile water and Buchner funnels with 100-mm diameters (Fisher Scientific, Pittsburgh, PA), and sterile 500-ml glass flasks with sidearms (Fisher Scientific) were stored at the same temperature as was the sample, which was analyzed—at either or 4 or 15°C—for 12 h until immediately prior to RNA extraction procedure. The laboratory in which the extraction was performed was cooled to 16°C the night before and maintained at this temperature during the extraction procedure. The room was kept at this temperature to prevent changes in mRNA expression of *E. coli* O157:H7 cells from their packaging environment. Bags containing inoculated shredded lettuce (35 g) were removed from storage (at either 4 or 15°C), immediately opened with a sterile scalpel, and added to stomacher bags (Fisher Scientific), each containing 100 ml of sterile deionized water, which was stored at either 4 or 15°C. The sample was then placed in a stomacher laboratory blender (Seward, Bohemia, NY), and homogenized for 2 min at 230 rpm. Bags were quickly removed from the stomacher and homogenate was

TABLE 1. *Sequences and concentrations of real-time PCR primers and probes used for virulence factors of interest (23)*

Primer or probe	Sequence (5′→3′)	Size (bp)	Concn (nM)
eaeL.2004RT (forward)	GTCTCAAACGCAAGCAACCA	101	600
eae-probe.2004RT (probe)	TCGTGCGACGATAAC		300
eaeR.2004RT (reverse)	CATCACTGACTGTCGCACTAACAGT		600
gndLT (forward)	GGTAATACCTTCTTCCAGGACACC	105	500
gnd-probe (probe)	CCGTGAGCTTTCTG		300
gnd-RT (reverse)	TAGTGCGCCCTCCTCACC		500
ehxAL.RT (forward)	GATATATTCCATGGCGCAGATG	77	200
ehxA-probe (probe)	TCGAAGGTAATTATGGT		300
ehxAR.RT (reverse)	CCATCATCGCCGTATAGTCG		200
ihaL.2004RT (forward)	GCATCTCACGGATGCACTTG	91	400
iha-probe.2004RT (probe)	CCGCTATGAACATCATG		300
ihaR.2004RT (reverse)	CAGATATGCACGCGGACTGA		400
rfbEL.RT (forward)	CAAGTCCACAAGGAAAGTAAAGATG	85	400
rfbE-probe (probe)	CACTTATTGGATGGTCT		400
rfbER.RT (reverse)	ATTCCTCTCTTTCCTCTGCGG		400
stx2L.(forward)	GATGTTTATGGCGGTTTTATTTGC	83	300
stx2-probe.June (probe)	TCTGTTAATGCAATGGC		300
stx2R.June (reverse)	TGGAAAACTCAATTTTACCTTTAGCA		300

immediately poured on to a 20-μm nylon net filter (Millipore, Billerica, MA) placed in a Buchner funnel, vacuum filtered, and collected in a 500-ml sidearm flask. A portion (25 ml) of the liquid homogenate collected in the flask was then transferred quickly into a sterile, 50-ml conical centrifuge tube containing 5 ml of stop solution composed of 95% ethanol (Sigma, St. Louis, MO) and 5% phenol (Sigma) at 4°C, and was placed on ice for 1 h. The remaining liquid homogenate was collected in separate 50-ml centrifuge tubes and stored on ice until microbiological analysis was performed. Samples were then transferred to sterile, 30-ml round-bottomed centrifuge tubes and centrifuged at 9,000 × *g* for 15 min at 4°C (Sorvall, Newington, CT). Supernatant was decanted, and the cell pellets were resuspended in 5 ml of supernatant. The resulting suspension was transferred to 1.5 ml-microcentrifuge tubes (Fisher Scientific) and centrifuged at 9,000 × *g* for 5 min at 4°C. Supernatants were completely removed from the cell pellet, and pellets were then stored at −20°C until RNA extractions and purifications were performed. RNA extractions from each cell pellet were performed by using a RiboPure RNA extraction kit (Ambion–Applied Biosystems, Austin, TX). After RNA extraction, samples were treated with TURBO DNase (Ambion) to remove residual DNA from samples. RNA samples were then quantified with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Samples were then stored at −20°C until real-time RT-PCR was performed. Three replicate RNA extractions were performed for each treatment at each temperature.

Quantitative real-time RT-PCR. Sequences of real-time RT-PCR primers and probes were the same as those sequences described by Rashid et al. *(23)* (Table 1), and were commercially prepared (Biosearch Technologies, Novato, CA). All probes were labeled with the FAM-490 fluorescent reporter marker. *gnd* is a housekeeping gene, used as a control because its expression correlates well with the growth rate of *E. coli* O157:H7 *(23)*. Real-time RT-PCR was conducted on an iCycler 7500 (BioRad, Hercules, CA). Real-time RT-PCR conditions were 50°C (15 min), and then 95°C (2 min), 95°C (15 s), and 54°C (45 s) for 40 cycles. A commercial RT-PCR kit was used to perform all real-time PCR assays (Superscript III One-Step RT-PCR system, Invitrogen, Carlsbad, CA). Concentrations of probes and primers in PCR reactions used for each gene are reported in Table 1. Standard curves for *eae, ehxA, iha, rfbE, stx*$_2$, and *gnd* were constructed by plotting cycle threshold counts (*x* axis) against log nanograms of RNA (*y* axis) by using 1, 5, 10, and 25 ng of total strain 52 *E. coli* O157:H7 total RNA (extracted from pure culture). For each inoculated lettuce sample examined, 10 ng of total RNA from each sample was analyzed in duplicate within each replicate experiment. Cycle threshold values of each virulence factor expressed on days 1, 3, 7, and 10 under each separate packaging and temperature condition evaluated were then used in regression equations to determine the relative amount (nanogram) of each virulence factor present. Amounts were expressed as ratios to the *gnd* value under each respective packaging and temperature condition.

Microbial analysis. On days 0, 1, 3, 7, and 10, filtered homogenates containing *E. coli* O157:H7 suspensions from each packaging treatment at each temperature were diluted appropriately in 0.1% peptone water and 0.1 ml were spiral plated (WASP, Don Whitley Scientific, Frederick, MD), in duplicate, on MACN and on TSA. Plates were incubated at 37°C for 24 h. Packaged lettuce samples that were not inoculated with *E. coli* O157:H7 were homogenized as described above. After homogenization, the homogenate was not filtered but collected and placed on ice until spiral plated on TSA and incubated at 37°C for 24 h. Uninoculated samples were not filtered as were inoculated samples because RNA extraction was not performed. Bacterial populations from all media were enumerated with a automated colony-counting instrument (Protocol, Synbiosis, Frederick, MD).

Gaseous analysis of inoculated and uninoculated packaged lettuce samples. Before microbial enumeration and RNA extraction from inoculated lettuce samples, O_2 and CO_2 levels in the headspace of the packages were determined on days 1, 3, 7, and 10, with a gas analyzer (Checkmate II, PBI Dansensor, Ringsted, Denmark). Samples were removed from either 4 or 15°C conditions individually, and a sponge septum was placed on the film; the needle of the instrument was then placed through the septum and into the plastic film until a reading was obtained. After the reading was measured, the needle was removed from the septum, the hole created was covered with electrical tape, and the package was placed at the appropriate storage temperature until RNA extraction and microbial analysis.

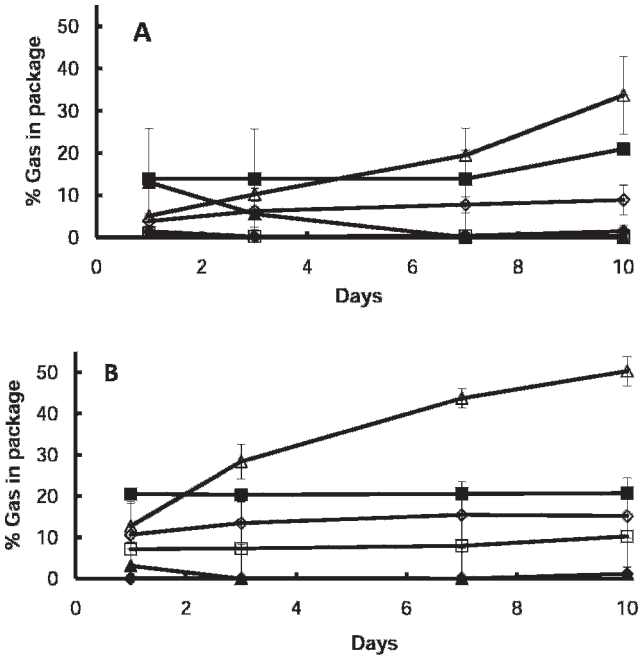

FIGURE 1. *(A) O_2 and CO_2 content of shredded lettuce packaged under treatment A (gas-permeable film, with 110 OTR, by using a N_2 flush to obtain 2% O_2 headspace and vacuum sealed), treatment B (near–ambient atmosphere conditions with, 110 OTR, with perforations), and treatment C (non–gas-permeable film, 0 OTR) at 4°C for up to 10 days. (B) O_2 and CO_2 content of packages stored under treatments A, B, and C at 15°C for 10 days. Treatment A contained O_2 (◆) and CO_2 (◇); treatment B contained O_2 (■) and CO_2 (□); and treatment C contained O_2 (▲) and CO_2 (△). Data represented are mean values from three replicate experiments.*

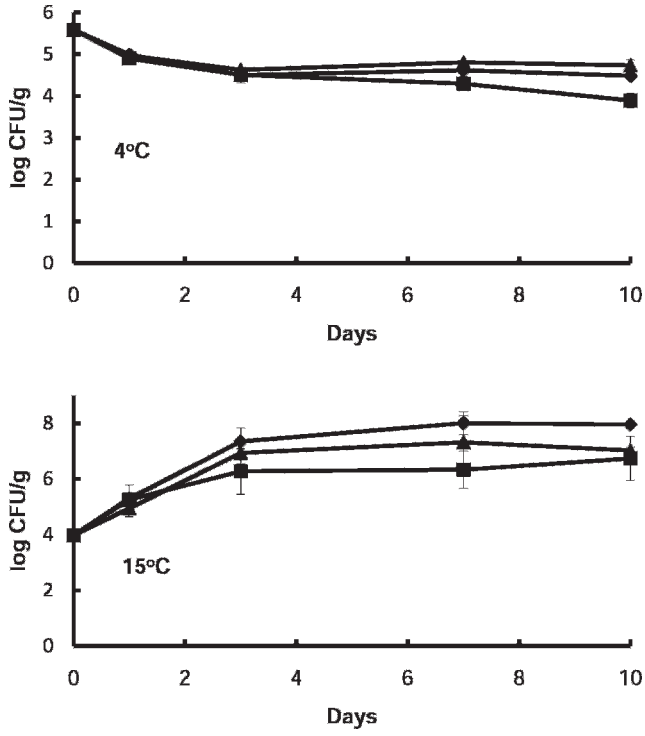

FIGURE 2. *Populations of* Escherichia coli *O157:H7 (log CFU per gram) on shredded lettuce, recovered on MACN, after storage at 4°C (top) and 15°C (bottom) on days 0, 1, 3, 7, and 10, packaged under: treatment A (◆; gas-permeable film, with 110 OTR, by using a N_2 flush to obtain 2% O_2 headspace and vacuum sealed), treatment B (■; near–ambient atmosphere conditions, with 110 OTR, with perforations), and treatment C (▲; non–gas-permeable film, 0 OTR). Populations represent mean values of three replicate experiments.*

Statistical analysis. *E. coli* O157:H7 and total aerobic population means from inoculated and uninoculated lettuce, respectively, under different treatments at 4 and 15°C on respective days, were determined. Using the least-significant different mean separation test in Statistical Analysis Software (version 9.0, SAS, Institute, Inc., Cary, NC), significant differences ($P < 0.05$) between populations means were determined. Ratios of respective virulence factor genes to *gnd* on specific days at the same temperatures across different packaging treatments were also compared with the same test. Three replicates for all experiments were performed.

RESULTS

Changes in package headspace gas composition. The atmospheric composition in lettuce packages varied among packaging treatments and storage temperatures, but the trend followed typical patterns of packaged fresh-cut lettuce subjected to similar treatments *(17–19)*. When stored at 4°C (Fig. 1A), the headspace O_2 level in packages receiving active modified atmosphere packaging (MAP; treatment A) reached equilibrium around 1.4 to 1.5% quickly, and it maintained this level throughout the 10-day storage period. Treatment B maintained an O_2 level near ambient air, due to microperforations in the packaging film. The O_2 levels of packages prepared with non–gas-permeable films (treatment C) declined rapidly, reaching near 0% O_2 on day 3. The trend observed with O_2 levels was reversed with respect to CO_2. For treatment A, CO_2 increased gradually during storage, reaching 8.9% on day 10 at 4°C. As expected, the CO_2 level in packages with microperforations had minimum accumulation of CO_2 (0.37% on day 10), while the CO_2 levels in packages prepared with gas-impermeable films accumulated rapidly, reaching 34% on day 10. Results from packages stored at 15°C resembled those stored at 4°C, with the treatment differences much more pronounced with respect to CO_2 levels (Fig. 1B).

Bacterial populations on shredded lettuce. Initial populations of *E. coli* O157:H7 on shredded lettuce at 4°C were 5.59 log CFU/g on day 0 for all treatments, and declined between 0.85 and 1.70 log CFU/g of *E. coli* O157:H7 over the 10-day storage period (Fig. 2A). *E. coli* O157:H7 populations on lettuce packaged under treatment B (4.30 and 3.89 CFU/g) were significantly ($P < 0.05$) lower than those populations on lettuce packaged under treatment C (4.81 and 4.74 log CFU/g) on days 7 and 10, respectively. Populations under treatment B (110 OTR, with microperforations) declined to the lowest levels (3.89 log CFU/g) among the three packaging treatments at 4°C after 10 days. *E. coli* O157:H7 populations on shredded lettuce stored at 15°C were 3.98 log CFU/g on day 0 and increased by 3.98 CFU/g (treatment A), 2.76 log CFU/g (treatment B), and 3.05 log CFU/g (treatment C) by day 10 (Fig. 2B).

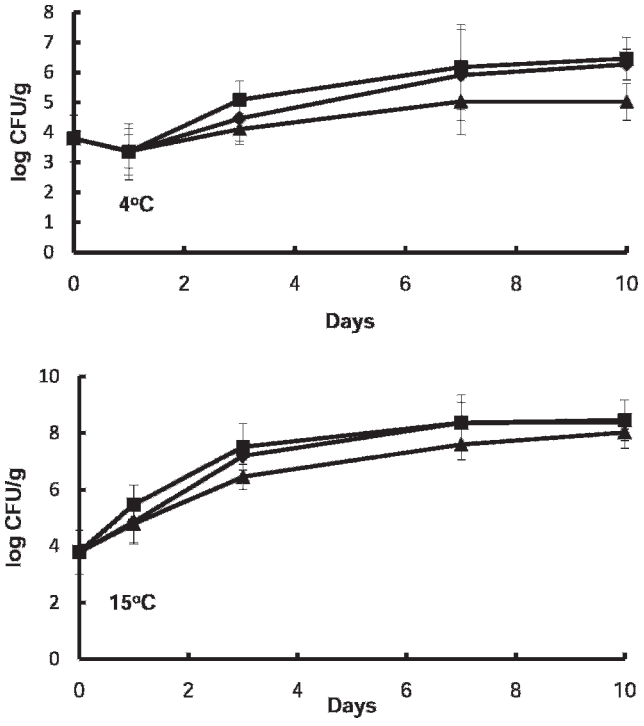

FIGURE 3. *Populations of total aerobic populations (log CFU per gram) on shredded lettuce, recovered on TSA, after storage at 4°C (top) and 15°C (bottom) on days 0, 1, 3, 7, and 10, packaged under treatment A (◆; gas-permeable film with 110 OTR, by using a N_2 flush to obtain 2% O_2 headspace and vacuum sealed), treatment B (■; near–ambient atmosphere conditions, with 110 OTR, with perforations), and treatment C (▲; non–gas-permeable film, 0 OTR). Populations represent mean values of three replicate experiments.*

Significant differences were observed only on day 10 between *E. coli* O157:H7 populations on shredded lettuce under treatment A (7.96 log CFU/g), and those packaged under treatment B (6.74 log CFU/g) and treatment C (7.02 log CFU/g). Total aerobic bacterial populations on uninoculated lettuce increased from 3.79 to 6.25 log CFU/g (treatment A), 6.46 log CFU/g (treatment B), and 5.01 log CFU/g (treatment C) after 10 days of storage at 4°C (Fig. 3A). Bacterial populations on uninoculated lettuce increased by between 4.23 and 4.56 log CFU/g for all treatments stored at 15°C (Fig. 3B).

Expression of *E. coli* O157:H7 virulence factors in packaged lettuce samples. Expressions of virulence factors of *E. coli* O157:H7 inoculated on shredded lettuce were measured in relation to the expression of *gnd* on each day at each temperature, and statistical differences ($P < 0.05$) were determined between treatments (Tables 2 and 3). Levels of expression of stx_2 in treatment C were significantly greater than were those levels in treatments A and B on day 10 at 4°C (Table 2). Levels of *eae, ehx, iha,* and *rfbE* were significantly greater under treatment C than were those levels in treatments A or B on day 1, but only levels of *rfbE* under treatment C were higher than they were under treatments A and B on day 3. For all virulence factors on all days, under no condition was expression of a virulence factor under treatment A greater than those virulence factors expressed when packaged under treatment B or C. In general, conditions under treatment B promoted higher expression of virulence factors than did other treatments at 15°C (Table 3). Expression of stx_2 was greater on inoculated lettuce packaged under treatment B than it was on inoculated lettuce packaged under treatment A or C on days 7 and 10. Expression of *eae* on inoculated lettuce packaged under treatment B was greater than expression under treatments A and C on days 3, 7, and 10 at 15°C. Packaging lettuce under treatment B also promoted high levels of expression of *rfbE* and *ehx* over treatments A and C after 7 and 10 days of storage at 15°C. Expression of *iha* on inoculated lettuce packaged under treatment B was significantly greater than expression under treatment C on days 1 and 3.

TABLE 2. *The expression of various* Escherichia coli *O157:H7 virulence factor genes relative to expression of the* gnd *gene on shredded lettuce packaged under three treatments and stored at 4°C*[a]

Virulence factor gene	Day	Treatment: A	B	C
stx_2	1	0.26 A[b]	0.26 A	0.34 A
	3	0.25 A	0.25 A	0.20 A
	7	0.59 A	0.35 A	0.68 A
	10	0.29 B	0.20 B	0.34 A
eae	1	0.92 B	0.98 B	1.50 A
	3	0.63 B	2.04 A	1.07 B
	7	—[c]	—	—
	10	—	—	—
ehx	1	0.41 B	0.36 B	0.59 A
	3	0.22 A	0.37 A	0.21 A
	7	0.26 A	0.18 A	0.45 A
	10	0.21 A	0.08 B	—
iha	1	2.73 B	1.87 B	4.09 A
	3	2.59 B	5.39 A	2.48 A
	7	—	—	—
	10	—	—	—
rfbE	1	0.71 B	0.64 B	0.75 A
	3	0.36 B	0.23 B	0.53 A
	7	0.49 B	—	1.02 A
	10	—	—	—

[a] Treatment A, gas-permeable film (110 OTR) flushed with N_2 to obtain 2% O_2 headspace, and vacuum sealed; treatment B, near–ambient atmosphere conditions in 110 OTR films with microperforations; treatment C, non–gas-permeable film, 0 OTR.

[b] Within columns ''Virulence factor gene'' and ''Day,'' mean values in the same row that are followed by different letters are significantly ($P < 0.05$) different. Mean values ($n = 3$) represent the ratio of the virulence factor gene to the *gnd* gene under conditions specified.

[c] —, mean values could not be determined for these virulence factors, due to a lack of amplification under these conditions.

DISCUSSION

Lettuce in this experiment was inoculated with *E. coli* O157:H7 and packaged under three separate conditions: an active MAP, treatment A (gas-permeable film in combina-

TABLE 3. *The expression of various* Escherichia coli *O157:H7 virulence factor genes relative to expression of the* gnd *gene on shredded lettuce packaged under three treatments and stored at 15°C*[a]

Virulence factor gene	Day	Treatment: A	B	C
stx$_2$	1	0.23 B[b]	0.25 B	0.50 A
	3	0.43 A	0.61 A	0.45 A
	7	0.35 B	0.58 A	0.37 B
	10	0.41 B	1.69 A	0.27 B
eae	1	0.30 A	0.29 A	0.35 A
	3	0.49 B	1.10 A	0.49 B
	7	0.44 B	0.80 A	0.32 B
	10	0.36 B	1.19 A	0.30 B
ehx	1	0.29 A	0.20 A	0.25 A
	3	0.33 A	0.33 A	0.38 A
	7	0.58 A	0.61 A	0.66 A
	10	0.49 B	1.68 A	0.39 B
iha	1	0.30 A	0.29 A	0.35 A
	3	0.49 B	1.10 A	0.49 B
	7	0.44 B	0.80 A	0.32 B
	10	0.36 B	1.19 A	0.30 B
rfbE	1	1.53 A	1.49 A	1.41 A
	3	3.99 A	2.66 B	3.44 B
	7	3.04 B	13.55 A	3.36 B
	10	2.85 A	3.03 A	3.26 A

[a] Treatment A, gas-permeable film (110 OTR) flushed with N_2 to obtain 2% O_2 headspace and vacuum sealed; treatment B, near–ambient atmosphere conditions in 110 OTR films with microperforations; treatment C, non–gas-permeable film, 0 OTR.

[b] Within columns ''Virulence factor gene'' and ''Day,'' mean values in the same row that are followed by different letters are significantly ($P < 0.05$) different. Mean values ($n = 3$) represent the ratio of the virulence factor gene to the *gnd* gene under conditions specified.

tion with an initial N_2 flush to accelerate establishment of the equilibrium of package atmospheres); treatment B, near–ambient atmosphere conditions (gas-permeable film, with microperforations); and treatment C (non–gas-permeable package film). The largest population reduction of *E. coli* O157:H7 was observed under treatment B (1.70 log CFU/g) after 10 days at 4°C, whereas treatments A and C displayed reductions of 0.85 and 1.10 log CFU/g, respectively, after 10 days. Population reductions under treatments A and C were similar to a 1-log reduction in *E. coli* O157:H7 counts on Romaine lettuce packaged under three different passive MAP conditions stored at 5°C for 10 days *(22)*. However, results observed in our study are not consistent with those results observed on Romaine lettuce inoculated with *E. coli* O157:H7 stored at 4°C under atmospheric conditions (no MAP), wherein populations declined by less than 0.50 log CFU/g over 9 days *(5)*. Types of lettuce and specific phyllosphere microflora might influence differences in *E. coli* O157:H7 counts. Previous work on spinach leaves reported that *E. coli* O157:H7 populations at an initial population of ca. 6 log CFU/g decreased slightly over 15 days at 4°C on spinach rinsed with tap water *(16)*. These same authors found that *E. coli* O157:H7 populations, inoculated on spinach leaves rinsed for 10 min in 12.5% sodium hypochlorite solution, increased by 0.81 log CFU/g over 15 days at 4°C. In our study, total aerobic populations on packaged uninoculated lettuce at 4°C under treatment B were significantly higher on day 10 (6.46 log CFU/g) than were those populations under treatment C (5.01 log CFU/g). The increased growth of total aerobic microbial populations under treatment B could indicate that the growth of psychrotrophic populations at 4°C influenced the decline of *E. coli* O157:H7 populations on inoculated shredded lettuce. Total aerobic bacterial population counts on lettuce inoculated with *E. coli* O157:H7 (data not shown) were similar to those observed on uninoculated shredded lettuce counts at both 4 and 15°C. The use of the selective media (MACN) to obtain *E. coli* O157:H7 from shredded lettuce might not have recovered physiologically stressed cells.

Populations of *E. coli* O157:H7 on lettuce packaged under all treatments at 15°C increased over 10 days, but they showed significant differences on day 10 between treatment A, and treatments B and C. Interestingly, *E. coli* O157:H7 populations on lettuce packaged under treatment B increased the least (by 2.76 log CFU/g), compared with treatments A (3.98 log CFU/g) and C (3.05 log CFU/g) at 15°C, while also decreasing the most (1.70 log CFU/g) at 4°C, compared with other treatments. These results could indicate that near–ambient air storage conditions might be more accommodating to the growth of background microflora, which are more detrimental to the survival and growth of *E. coli* O157:H7 at either 4 or 15°C, than are the typical commercial MAP conditions (treatment A) or high CO_2–low O_2 conditions (treatment C). At 15°C, total aerobic populations on uninoculated lettuce under treatment B after 10 days (8.45 log CFU/g) were not much different than were those populations under treatment A (8.37 log CFU/g) or C (8.01 log CFU/g). However, it is possible that packaging conditions under treatments B and C promoted the growth of microorganisms more inhibitory to the growth of *E. coli* O157:H7 than under treatment A. Levels of O_2 and CO_2 in packaging treatment B ranged from 13.9 to 20.1%, and 1.0 to 0.37%, respectively, when stored at 4°C; at 15°C, gas levels were between 20.5 and 20.7% O_2, and 7.2 and 10.3% CO_2 at 15°C. The commercial processing of the shredded lettuce also might have decreased non–*E. coli* O157:H7 bacteria populations on lettuce, which could have affected both the decline and growth of *E. coli* O157:H7 at 4 and 15°C in our study. Another study indicated that populations of *E. coli* O157:H7 on Romaine lettuce stored at 15°C under atmospheric conditions decreased slightly over 9 days *(5)*. This lack of growth at 15°C might be attributed the initial level (6.5 log CFU/g) recovered from Romaine lettuce in their study. A slight decrease in this high population on Romaine lettuce could indicate that 6.5 log CFU/g was the highest population of *E. coli* O157:H7 that nutrients provided by cut Romaine lettuce could maintain under these storage conditions. Cut iceberg lettuce inoculated with 3 log CFU *E. coli* O157:H7/g and packaged under aerobic atmospheric conditions stored at 10°C for 14 days revealed no change in *E. coli* O157:H7 populations throughout the storage period *(12)*. In our study, the increase

in *E. coli* O157:H7 populations could be due to the lower initial *E. coli* O157:H7 population (3.98 log CFU/g) on day 0, allowing the population of cells to grow to higher populations on cut iceberg lettuce at 15°C. Cut Romaine lettuce stored under three different passive MAP conditions at 25°C supported a 4-log CFU/g increase in *E. coli* O157:H7 populations *(22).* Spinach rinsed briefly in tap water and then inoculated with *E. coli* O157:H7 stored at 10°C for 15 days under atmospheric conditions supported a much smaller increase (0.76 log CFU/g) in populations than spinach washed in 12.5% sodium hypochlorite (3.94 log CFU/g) in *E. coli* O157:H7 populations *(16).* Our results indicate that near–ambient air storage conditions, combined with abusive temperatures, reduce the ability *of E. coli* O157:H7 to grow on the surface of shredded lettuce.

Commercial RNA stabilization reagents were not used in our study because of their potential effect to induce stress responses in bacterial cells *(2).* Populations of *E. coli* O157:H7 on shredded lettuce inoculated for storage at 4°C were higher than were those populations on shredded lettuce stored at 15°C, ensuring that sufficient bacterial RNA could be extracted from *E. coli* O157:H7 cells at nongrowth temperatures. Expression of virulence factors were normalized to *gnd,* a gene that encodes for the third gene in the pentose–phosphate pathway *(23).* The presence of *gnd* is specific to *E. coli* O157:H7 and is correlated with the growth rate of the organism. Because our extraction procedures isolated total bacterial RNA from samples (including *E. coli* O157:H7 RNA), the use of *gnd* allowed for a specific *E. coli* O157:H7 gene to be used as an internal standard in all samples. Because of the lack of *E. coli* O157:H7 growth at the 4°C storage temperature, the presence of mRNA presumably declined throughout the storage period and did not permit the extraction of high-quality mRNA under certain conditions. With the exception of stx_2, expression of all virulence factors under treatment C was significantly greater than it was under treatments A and B after 1 day of storage at 4°C, although the increases in these levels were not dramatic. There was a greater than twofold difference in the expression of *iha* under treatment C on day 1 (4.09), compared with treatment B (1.87), and for *rfbE* under treatment C (1.02), compared with treatment A (0.49) at day 7 on 4°C. Both of these genes play a role in the attachment of *E. coli* O157:H7 cells to human and animal intestinal cells, and it is hypothesized that they allow *E. coli* O157:H7 to persist in intestinal tracts *(23, 26).* Our results do not show a demonstrative increase in expression of stx_2 over the course of 4°C storage under the three packaging conditions examined here; this differs from results reported by others, who stated relative increases in the levels of expression of stx_2 on Romaine lettuce stored at 4°C for 9 days under atmospheric conditions *(5).* Expression of stx_2 was significantly greater on day 5 than it was on day 0 or day 10 on inoculated spinach leaves stored at 4°C when leaves were rinsed in tap water *(16).* In these studies, the normalization of *stx* genes to different housekeeping genes and differences in commodities and packaging conditions might reflect the differences in relative gene expression values, as compared with results presented here. Expression of the *eae* gene in our study did not increase over 3 days under any packaging treatment at 4°C, similar to results reported by others *(5).*

Our study uncovered significant differences in the expression of all *E. coli* O157:H7 virulence factors on inoculated iceberg lettuce stored at 15°C, based on packaging treatments. Expression of virulence factor under treatment B was greater than expressions observed under treatments A and C were in all cases on varying days. Under treatment B, the expression of all virulence factors increased over 10 days of storage at 15°C, whereas the same trend was not observed under packaging treatments A and C. Expression of stx_2 under treatment B was fourfold higher than it was under treatments A, and sixfold higher than it was under treatment C after 10 days of storage at 15°C. Results observed in our study are similar to those observed on rinsed spinach stored at 10°C, in which expression of stx_2 increased throughout the entire 10-day storage period *(16).* Previous work has shown that stx_2-positive (wild type) *E. coli* O157:H7 infections in infant rabbits caused longer duration and severity of diarrhea, compared with a stx_2-deficient strain (isogenic mutant of wild type) of *E. coli* O157:H7 *(24).* In this same work, the direct intragastric addition of Stx2 to infant rabbits still caused inflammation and diarrhea, and altered the host response on a cellular level, as compared with the response to stx_2-deficient strains. The increased expression of stx_2 on packaged lettuce under treatment B stored at 15°C might lead to more Shiga toxin production by *E. coli* O157:H7 on lettuce, thus leading to possible foodborne intoxication before or without colonization of the host.

The expression of *eae* on packaged lettuce under treatment B was ca. twofold higher than it was under treatments A or C after 3 days of storage at 15°C, and ca. threefold higher than other treatments after 10 days at 15°C. In previous work, the expression of *eae* on inoculated lettuce stored under atmospheric conditions at 15°C for 9 days did not increase *(5)* as it did in our study under treatment B. The *eae* gene is a critical factor for *E. coli* O157:H7 attachment and colonization of intestinal cells, and it is essential to the formation of attachment and effacement lesions in hosts *(13).* Greater expression of the *eae* gene on lettuce might allow for more rapid colonization of gastrointestinal tracts. *E. coli* O157:H7 lacking the *eae* gene was unable to colonize intestinal cells in infant rabbits, resulting in no diarrhea in these animals *(24).* Expression of *ehxA* on lettuce packaged under treatment B was at least threefold higher than it was under treatment A or treatment C, after 10 days of storage at 15°C. The role of *ehxA* in human infections is unclear: It is not required for colonization *(24),* although its expression is upregulated in human infection over bovine colonization *(23).* Enterohemolysin (a siderophore) production by EHEC strains was enhanced under iron-limiting conditions on blood agar media incubated at 37°C for 16 h in 8% CO_2, 52% N_2, and 40% H_2 and 21°C for 6 h in air *(10).* In treatment B at 15°C, concentrations of CO_2 were between 7 and 10%, which may have enhanced the expression of *ehxA*. However, CO_2 concentrations in treatments A and C at 15°C were higher

than was the concentration in treatment B, demonstrating that CO_2 might not be the only determinative factor of *ehxA* expression on packaged shredded lettuce. Virulence factors involved in attachment and persistence of *E. coli* O157:H7 (encoded by *iha* and *rfbE*) were also upregulated in lettuce packaged under treatment B, as compared with treatments A and C. The *iha* gene is significantly upregulated in bovine infections when compared with human infections of *E. coli* O157:H7, while *rfbE* appears to be upregulated in human infections *(23)*. *E. coli* O157:H7 mutants lacking the *rfbE* gene did not persist as long as did wild-type cells in the intestines of streptomycin-treated mice *(26)*, indicating that *rfbE* might play a role in the attachment and colonization of intestinal cells. Other work has shown that *rfbE*-deficient *E. coli* O157:H7 adhered in greater numbers than do wild-type strains to HeLa cells *(1, 4)*, indicating expression of the *rfbE* gene could be more crucial during intestinal colonization than it is during attachment to epithelial cells.

A storage temperature of 15°C could be the extreme of temperature abuse that product could encounter under commercial, retail, or home storage. Conditions used in treatment B might resemble more closely the conditions after the opening of sealed packages of lettuce and subsequent storage of the remaining servings. Disruption of the integrity of the package might also lead to a lack of atmospheric and temperature control during transport or retail display. However, these results emphasize the need to keep shredded, bagged lettuce under proper refrigeration conditions throughout all stages of production to consumption. It should be noted that treatment A, which most closely resembled commercial packaging conditions in our study, did not promote increased virulence of *E. coli* O157:H7 on shredded lettuce when compared with treatments B and C at either 4 or 15°C.

Expression of virulence factors might not be the sole reason for the increased virulence of *E. coli* O157:H7 infections through the consumption of contaminated lettuce. Storage of inoculated shredded lettuce under MAP conditions at 15°C for 8 days promoted the induction of acid resistance in *E. coli* O157:H7 *(11)*, increasing the likelihood that this organism can survive the gastric acid challenge encountered in the human gut. Others have determined that the *E. coli* O157:H7 strains involved in the 2006 spinach and lettuce outbreaks are associated with genetically divergent subpopulations of *E. coli* O157:H7 that have acquired critical factors that might result in illness more severe *(20)*. This subpopulation (clade 8) is more likely to possess a combination of stx_2 and stx_{2c} genes, and might constitute an increasing percentage of *E. coli* O157:H7 strains responsible for outbreaks in the United States *(20)*. If *E. coli* O157:H7 strains involved in leafy green outbreaks are more likely to cause illness more severe in humans, based on a combination of *stx* genes, then the understanding of factors that might promote or diminish the expression of stx_2 genes under MAP conditions used for shredded lettuce becomes more critical.

In conclusion, near–ambient air conditions (treatment B) supported lower populations of *E. coli* O157:H7 on inoculated shredded iceberg lettuce when stored at 4°C, as compared with active MAP conditions (treatment A) or high CO_2–low O_2 conditions (treatment C). However, these conditions that promoted the inhibition of *E. coli* O157:H7 also supported higher levels of virulence factor expression when compared with treatments A and C at 15°C. Conditions representing commercial packaging conditions (treatment A) did not promote increased virulence expression at 4 or 15°C. Results reported here support proper refrigerated storage of bagged leafy greens. Our work shows that packaging conditions can affect the expression of *E. coli* O157:H7 virulence factors on shredded lettuce. The increased expression of stx_2 under these conditions, coupled with abusive temperatures and virulent *E. coli* O157:H7 strains, could contribute to the severity of illness observed from the consumption of bagged, fresh-cut leafy greens.

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